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Development of a Monoclonal Antibody-Based Broad-Specificity ELISA for Fluoroquinolone Antibiotics in Foods and Molecular Modeling Studies of Cross-Reactive Compounds

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A competitive indirect enzyme-linked immunosorbent assay (ciELISA) using monoclonal antibodies (Mabs) having broad specificity for fluoroquinolone (FQ) antibiotics is described. Four FQs, ciprofloxacin (CIP), enrofloxacin (ENR), norfloxacin (NOR), and ofloxacin (OFL), were conjugated to bovine serum albumin for immunogens and to ovalbumin for coating antigens. A Mab C4A9H1 raised against the CIP hapten exhibited high cross-reactivity (35−100%) with 12 of 14 FQs and detected these FQs in a ciELISA below their maximum residue levels (MRLs) with good sensitivity at 50% binding inhibition (IC_{50}). The quantitative structure−activity relationship (QSAR) between Mab C4A9H1 and various FQs by comparative molecular field analysis (CoMFA) showed a high predictive ability with a cross-validation q^2 value of 0.866. Using a simple purification process and the broad-specificity ciELISA adapted for analysis of FQs in chicken muscle, chicken liver, honey, shrimp, and whole egg samples demonstrated recoveries of 60−93% for CIP, ENR, NOR, OFL, flumequine, and danofloxacin.

Fluoroquinolone (FQ) antibiotics are derived from the quinolone nalidixic acid by introduction of the piperazine moiety at position 7 and a fluorine atom at position 6 (Figure 1). FQs are comparatively more effective against Gram-negative bacteria and some Gram-positive bacteria than quinolone antibiotics.[1] FQs were introduced for human use in Europe and the United States in the mid-1980s and were approved for livestock treatment in the mid-1990s[2] and since then have became the most commonly prescribed antibiotics.[3] However, due to the extensive use of FQs in the animal industry and aquaculture, residues of these drugs are a major concern, especially for those who are allergic to these drugs. Furthermore, the emergence of food-borne bacteria that are resistant to FQs has recently prompted formulation of regulatory rules for the use of these antibiotics in food animals.

The resistance to FQs in U.S. human *Campylobacter jejuni* isolates was observed to increase from zero in 1991 to elevated levels (13%) soon after the introduction of FQ use in commercial chicken production. A similar observation was reported from Europe.[4] In the case of *Salmonella*, the most frequently isolated bacteria from food-borne outbreaks of gastroenteritis, reduced susceptibility to the FQs has increased in both humans and animals, especially in Europe, Southeast Asia, and the Indian subcontinent.[5] Recently, failures of typhoid fever treatment with FQs and increased minimum inhibitory concentrations (MICs) of FQs required against *S. enterica* Typhi[6] and *S. enterica* Paratyphi A are of concern.[7] It is estimated that globally there are 16 million new cases of typhoid fever each year, which result in 600 000 deaths.[8] To minimize the risk of FQ exposure to humans during consumption of animal products and to preserve the efficacy of FQs, maximum residue limits (MRLs) have been established for several FQs by the European Commission[1] and the China Ministry of Agriculture (CMA) (no. 278.2003.5.22). Therefore, there is a growing demand for easy, rapid, and economical methods to monitor FQ residues in foodstuffs.

The most commonly used analytical technique for determination of FQs is liquid chromatography with fluorescence detec-

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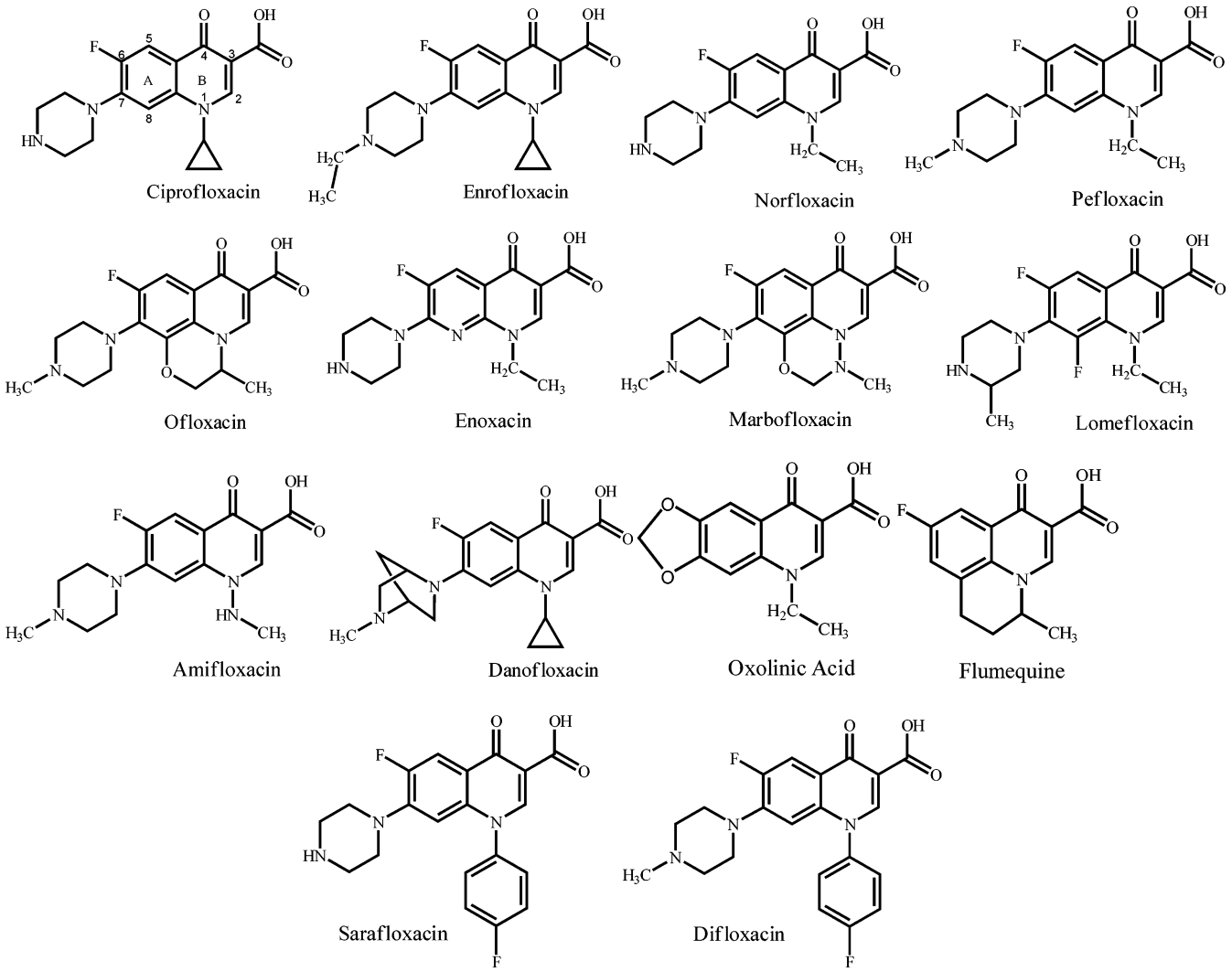

Figure 1. Fluoroquinolone structures.

tion.[9,10] Confirmatory analysis using liquid chromatography-tandem mass spectrometry has been reported.[11,12] Capillary electrophoresis has also proved to be an effective separation tool for FQs.[13] However, many of the instrumental methods used to monitor these residues are time-consuming, solvent intensive, and costly; therefore, chromatographic and mass spectrometry methods are not practical for screening large numbers of food samples for FQs. Analytical methods with increased throughput and reduced environmental impact are required. The enzyme-linked immunosorbent assay (ELISA) was widely employed because of its sensitivity of detection and ease of use.[14]

Several papers have reported the development of an immunoassay for the determination of specific FQs;[15−20] however, the current trend is to develop assays capable of measuring multiple targets in a single test or a generic assay. The progress of immunoassay development is constrained by the availability of antibodies with appropriate affinity and specificity characteristics.[21] An earlier attempt to obtain antibodies for the development of a FQ generic immunoassay utilized the secondary amine on the piperazinyl moiety at carbon-7 (Figure 1) of the FQ norfloxacin for an attachment site to a carrier protein.[19] Through this approach the researchers produced polyclonal antibodies, nine FQs were detected with a cross-reactivity of 6% or more. Recently, Huet et al. developed a generic immunoassay for a set of 11 FQs with IC_{50} values ranging from 0.21 to 1.52 ng mL^{-1} and with cross-reactivities over 14%,[22] but a generic immunoassay for FQs based

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on the use of a monoclonal antibody (Mab) has not been described. Some of the specific immunoassays based on polyclonal antibodies did possess some generic characteristics,[15,18] and the immunogens were prepared by directly conjugating the carboxyl group at carbon-3 (Figure 1) to a carrier protein. This conjugation strategy appeared to be a promising way to develop a generic immunoassay for FQs. In this paper we describe the development of a generic ELISA for the detection of 12 FQs. This generic ELISA was used to quantify six FQs in five different food matrices. We also use molecular modeling and comparative molecular field analysis (CoMFA) to identify structural features of 14 FQs with respect to different antibody affinities.

EXPERIMENTAL SECTION

Reagents and Chemicals. Bovine serum albumin (BSA), ovalbumin (OVA), isobutyl chloroformate, tributylamine, 3,3′,5,5′-tetramethylbenzidine (TMB), *N*-hydroxysuccinimide (NHS), and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO). The myeloma cell line SP2/O was a gift from Professor Jixun Zhao (China Agricultural University, Beijing, P.R.C.). Dulbecco's modified Eagle media (DMEM) used for cell culture was obtained from Huamei (Beijing, P.R.C.). Incomplete Freund's adjuvant (IFA), complete Freund's adjuvant (CFA), fetal calf serum, supplements, and a dipstick format mouse Mab isotyping kit (goat antimouse IgG−HRP) were obtained from Gibco BRL (Carlsbad, CA). Acetonitrile was obtained from Dikma (Beijing, P.R.C.). All other chemicals and solvents were of analytical grade or better and were obtained from Beijing Chemical Reagent Co. (Beijing, P.R.C.). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA). Enrofloxacin (ENR) (purity, 100%), ciprofloxacin (CIP) hydrochloride (100%), norfloxacin (NOR) (99.6%), flumequine (FLU) (99.5%), pefloxacin methanesulphonate (PEF) (99.9%), sarafloxacin (SAR), and difloxacin (DIF) were purchased from the China Institute of Veterinary Drug Control (Beijing, P.R.C.). Ofloxacin (OFL) (≥99.0%), lomefloxacin (LOM) (99.8%), enoxacin (ENO) (>99.0%), danofloxacin (DAN) (≥99%), amifloxacin (AMI) (≥99%), oxolinic acid (OA) (≥99%), and marbofloxacin (MAR) (≥99%) were purchased from Sigma Chemical Co. (St. Louis, MO).

Instrumentation. Polystyrene microtiter plates were purchased from Beijing Wanger Bio. Tech. Co. (Beijing, P.R.C.). The ELISA plate reader was obtained from TECAN Inc. (Durham, NC). The UV−vis spectrometer was obtained from Shanghai Analytical Instrument (Shanghai, P.R.C.).

Standard Solutions. Standard stock solutions (100 μg mL^{-1}) of antibiotics were prepared by dissolving an appropriate amount of each standard in 0.03 M sodium hydroxide. The individual stock solutions were stored at 4 °C in amber glass bottles and were stable for at least 3 months. Working standards (0.01, 0.03, 0.09, 0.27, 0.9, 3, 9, 27, 81, 240, and 720 ng mL^{-1}) of each antibiotic was prepared by diluting the stock solution in assay buffer.

Buffers and Solution. The following buffers were used in the competitive indirect enzyme-linked immunosorbent assay (ciELISA): (1) coating buffer was 0.05 M carbonate buffer, pH 9.6; (2) blocking buffer consisted of 0.02 M phosphate-buffered saline (PBS), pH 7.4, 0.5% casein, 1% BSA, 0.01% NaN_3, and 0.05% Tween 20; (3) washing buffer was PBS with 0.05% Tween 20; (4) antibody dilution buffer was PBS, containing 0.05% Tween 20, 0.1% albumin, and 0.01% NaN_3; (5) enzyme-labeled secondary antibody dilution buffer was antibody dilution buffer containing 10% albumin; (6) substrate was 0.1% TMB and H_2O_2 in 0.05 M citrate buffer, pH 4.5; (7) 2 N H_2SO_4 was the stopping reagent.

Synthesis of Immunogens and Coating Antigens. The immunogens were prepared by conjugating haptens NOR, CIP, ENR, and OFL to BSA based on a mixed anhydride method[23] with the following modifications: the hapten (100 mg) was dissolved in 10 mL of dimethylformamide (DMF), and the solution was cooled with cold ethanol (4 °C). Then 30 μL of triethylamine and 40 μL of isobutyl chloroformate were added. The resulting mixed anhydride solution was stirred at room temperature for 20 min before 3 mL of 5 mg mL^{-1} BSA in carbonate buffer (pH 9.6) was added dropwise followed by continuous stirring at room temperature for 6 h. The hapten−BSA conjugates were dialyzed against PBS (pH 7.0) at 4 °C for 72 h, and then were characterized by UV−vis spectrometry.[24]

The coating antigens were prepared by conjugating the same four haptens above to OVA using the NHS ester method previously described[25] with modifications: the hapten (15 mg) was dissolved in DMF (5 mL), and the solution was cooled with cold ethanol (4 °C). NHS (20 mg) and EDC (20 mg) were added to the hapten solution, and the mixture was stirred at room temperature overnight. OVA (15 mg) was dissolved in 5 mL of carbonate buffer (pH 8.0) and added dropwise to the active NHS solution with continuous stirring and further stirred at room temperature for 4 h. The hapten−OVA conjugates were dialyzed against PBS (pH 7.0) at 4 °C for 72 h, and then characterized with UV−vis spectrometry.[24]

Production of Monoclonal Antibodies. The procedures used for the production of Mabs were similar to those described by Zhang et al.[26]

Immunization. Female BALB/c mice (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, P.R.C.) were immunized subcutaneously with 100 μg of either NOR−BSA, CIP−BSA, OFL−BSA, or ENR−BSA in 0.25 mL of 0.85% NaCl and 0.25 mL of CFA. Two and four weeks after the initial injection, animals were boosted with 100 μg of the same conjugate in IFA. One week after the last injection, mice were tail-bled, and the serum antibody was monitored by ciELISA using the corresponding NOR−OVA, CIP−OVA, OFL−OVA, or ENR−OVA conjugate as the coating antigen.

Cell Fusion. Cell fusion procedures were carried out as previously described.[27] The mouse that had the highest polyclonal antibody titer was sacrificed, and splenocytes were fused with SP2/0 myeloma cells using polyethylene glycol (PEG 4000). The fused cells were propagated in HAT medium, plated in six 96-well microculture plates, and then were screened for specific Mabs using the ciELISA.

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Hybridoma Selection and Cloning. Eight to ten days after cell fusion, culture supernatants were screened for the presence of antibodies that recognized the analyte. Supernatants showing an inhibition of >80% in the presence of 1000 ng mL^{-1} hapten compared to the absence of analyte were considered to be derived from high-affinity antibody-secreting hybridomas. The cultures showing the highest inhibition by NOR, CIP, OFL, and ENR were cloned twice by limiting dilution. The clone with the highest inhibition was cloned twice prior to ascites production. The antibody was purified using saturated ammonium sulfate and stored at −20 °C in 50 μL aliquots. The concentration of antibody was measured according to the following formula:

$$\text{protein concentration (mg mL}^{-1}) = 1.45\text{OD}_{280\text{nm}} - 0.74\text{OD}_{260\text{nm}}$$

OD is the optical density.

Mab isotype was determined using a Mab isotyping kit.

ELISA Methods. The general ELISA protocols were similar to those used in this simplified ciELISA approach in comparison to the conventional ciELISA,[28] and our optimized methods are described as follows: Polystyrene 96-well microtiter plates were coated with NOR−OVA, CIP−OVA, OFL−OVA, or the ENR−OVA conjugate (100 μL/well) and incubated at 37 °C for 2 h, and then at 4 °C overnight. The plates were washed three times with washing buffer, and then blocked with blocking buffer (300 μL/well) at 37 °C for 1 h. Antibody ascites (50 μL/well), various concentrations of analytes (50 μL/well) or samples (50 μL/well), and goat antimouse IgG-HRP (1:2000 in PBS, 100 μL/well) were simultaneously added to the conjugate-coated microplates and incubated at 37 °C for 1 h. Following incubation the plates were washed three times with washing buffer. The substrate solution (100 μL/well) was added and incubated at 37 °C for 30 min before the enzymatic reaction was stopped by adding 2 N H_2SO_4 (100 μL/well). The absorbance (*A*) of each well was measured at 490 nm by the ELISA plate reader. The ciELISA was used to determine each Mabs affinity and cross-reactivity.

Cross-Reactivity Determination. Stock solutions of different FQs were prepared as described in the Standard Solutions section. Standard curves were prepared in assay buffer, and each IC_{50} value was determined in the competitive experiment described above. The cross-reactivity values were calculated according to the following equation:

$$(\text{IC}_{50}(\text{hapten, ng mL}^{-1})/\text{IC}_{50}(\text{FQ analogs, ng mL}^{-1})) \times 100\%$$

Sample Preparation. Chicken muscle, chicken liver, shrimp, honey, and whole egg were purchased from a local market. Prior to running the assay, all solutions were diluted with assay buffer to the optimum concentration.

Chicken Muscle, Chicken Liver, and Shrimp. Homogenized chicken muscle, chicken liver, or shrimp were weighed (3.0 g) into 50 mL tubes, and 9 mL of 0.1 M sodium hydroxide−acetonitrile (1:10) was added, and the mixture was agitated on a shaker for 10 min. The samples were centrifuged at 3000*g* for 10 min at 15 °C, and the supernatants (4 mL) were added to an equal volume of 0.02 M PBS (pH 7.2) and mixed with dichloromethane (8 mL) for 10 min. The mixture was centrifuged at 3000*g* for 10 min at 15 °C, and the lower organic phase was dried using nitrogen gas at 50 °C. The organic phase residue was redissolved with assay buffer and washed twice with 1 mL of hexane, and 50 μL of this buffer solution was used in the ELISA.

Honey. Honey samples (1 g) were dissolved with 2 mL 0.05 M PBS (pH 7.2), dichloromethane (8 mL) was added, and the solution was mixed for 5 min and centrifuged at 3000*g* for 5 min at 15 °C. Four milliliters of the organic phase was dried using nitrogen gas at 50 °C. The residue was redissolved with 2 mL of 0.05 M PBS, and 50 μL was used in the ELISA.

Whole Egg. Homogenized whole egg (2 g) was extracted with 8 mL of acetonitrile for 10 min and then centrifuged at 3000*g* for 10 min at 15 °C. The supernatant (2 mL) was dried using nitrogen gas at 60 °C. The residue was redissolved with 1 mL of 0.02 M PBS and washed twice with 1 mL of hexane, and 50 μL of the washed buffer was used for the ELISA.

Curve Fitting and Statistical Analysis. A four-parameter logistic equation was used to fit the immunoassay data. Calculations were performed using OrginPro 7.5 software (OriginLab Corporation, Northampton, MA). The IC_{50} value, assay dynamic range, and the limit of detection (LOD) served as criteria to evaluate the ciELISA. These different characteristics represent the analyte concentrations obtained at 50%, between 20% and 80%, and at 90%,[29] respectively.

Molecular Modeling and CoMFA Calculation. All molecular modeling calculations were conducted with Sybyl 7.0 (Tripos Inc., U.S.A.) running on the Silicon Graphics Fuel Visual workstation (Silicon Graphics Inc., U.S.A.).

Energy Minimization. Minimum energy conformations of all structures were calculated using the Minimize module of Sybyl 7.0. The force field was MMFF94[30] with an 8 Å cutoff for nonbonded interactions, and the atomic point charges were also calculated with MMFF94. Minimizations were achieved using the steepest descent method for the first 100 steps, followed by the BFGS calculation method (named for its originators; approximates the inverse of the Hessian matrix)[31] until the root-mean-square (rms) of the gradient became less than 0.005 kcal/(mol·Å).

Determination of Surface Properties. Molecular surfaces were calculated in the Create Surface module using the Accelrys DS Visualizer. Surface coloring was based on "electrostatic potential", display style was "solvent", and the probe radius was 1.4 Å.

Calculation of CoMFA. For CoMFA calculations, the alignment molecules were placed in a three-dimensional (3D) cubic lattice with 2 Å spacing. The default sp^3 carbon atom with +1 charge was selected as the probe atom for the calculation of the steric (Lennard-Jones 6−12 potential) and electrostatic fields (Coulombic potential) around the aligned molecules with a distance-dependent dielectric constant at all lattice points. The column filtering was set to 1.0 kcal/mol. Values of steric and electrostatic energies were

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truncated at 30 kcal/mol. The regression analysis was carried out using the partial least-squares (PLS) method. Regression analysis was performed using the leave-one-out method. The optimum number of components to be used in the conventional analysis was defined as that which yielded the highest cross-validated q^2.

RESULTS AND DISCUSSION

Haptens. For most immunoassays, the key step in preparing the immunoassay is the design and synthesis of the optimum hapten. In preparing broad-specificity antibodies against veterinary drugs such as the sulfonamides[32] and other small molecules,[33−35] complicated multistep hapten syntheses were used, and sometimes several haptens were required. Fortunately, while producing a broad-specificity antibody to FQs, an intricate hapten synthesis was not necessary due to an active carboxylic group presented at position 3 in ring B of the FQs (Figure 1), which was directly conjugated to a carrier protein. Our aim was to produce antibodies that could recognize most FQ analogs and develop an immunoassay that could determine the total array of FQs found in food samples. Previous studies reported that NOR and SAR conjugated by different means to carrier proteins and used as immunogens resulted in antibodies that detected at least five FQs with cross-reactivities of more than 10%.[18,19] Recently, NOR and SAR were used as haptens to prepare antibodies for a generic FQ immunoassay.[22] In the study, a carboxylic acid group was introduced on the piperazinyl ring situated at position 7 of ring A. This compound still retained the carboxylic acid at position 3 of ring B, which is common in FQs. It is evident that one or more FQs can be used as a hapten to produce a broad-specificity antibody, and we suggest that NOR and CIP maybe desirable haptens for the production of a generic antibody based on the following four assumptions: (i) The FQ structures all possess a nitrogen-containing, six-membered heterocyclic aromatic ring, a ketone group at position 4 of ring B, and many of the FQs have a piperazinyl ring at position 7 of ring A. All of these common structural features in many FQs, a carboxylic acid group, a ketone group, and a piperazinyl group, would be helpful in obtaining a generic antibody. (ii) In the two selected FQ molecules, there is no substituent present on the piperazinyl ring, and therefore these two molecules closely mimic the common moiety in the FQs, while there is an alkane substituent (ethyl group for NOR and cyclopropyl group for CIP) at position 1 of ring B. These groups at position 1 of ring B would be expected to have only a weak antigenic effect in the animal immune system. (iii) Although the carboxylic acid group at position 3 of ring B was used to conjugate to the carrier protein, the absence of the carboxylic acid should have limited influence on the resulting generic antibody except for the recognition of OA and FLU. Thus, the greater portion of the molecules containing the conserved structure in this class of drugs would be presented to the immune system. (iv) The structural aspects that appear different in most FQ analogs are in the piperazinyl ring substituents or at position 1 of ring A, as is the case between CIP and NOR. Since these differences are usually small, the alkyl groups exert a limited influence on antibody binding. The chemical structure and the electronic distribution of the target analyte does play an important role in the formation of high-quality antibodies.[36] Also, the antibody's structural binding characteristics may be driven by the resulting final 3D conformation of the hapten resulting from the immunized animal's body temperature.[37] Thus, to increase the probability of obtaining the desired generic antibody, the four selected haptens NOR, CIP, ENR, and OFL were conjugated to BSA with the mixed anhydride method and used for immunogens, and these haptens were also conjugated to OVA with the NHS method and used as coating antigens. Spectral analysis of the hapten−protein conjugates indicated that NOR, CIP, OFL, and ENR were incorporated onto BSA at 25, 19, 26, and 23 mol per mol of protein, and the amount incorporated onto OVA was 18, 17, 22, and 20 mol per mol of protein, respectively.

Monoclonal Antibody Production. Four mice were used for the fusion experiments, one mouse for each selected hapten conjugate. Mouse 5, 23, 36, and 54 were immunized with CIP−BSA, NOR−BSA, ENR−BSA, and OFL−BSA, respectively. Hybridomas demonstrating a strong positive response in the absence of analyte were reassayed after diluting the culture supernatant to ensure that antibodies were not in excess. Although most of the hybridomas did not behave competitively under the screening conditions, a low but significant number of them were capable of recognizing their corresponding FQ haptens with high affinity when assayed in ELISA wells coated with the corresponding coating antigens. From these fusions using four immunizing haptens, nine hybridomas secreting antibodies with high affinity to their corresponding FQs were obtained: a total of two, three, two, and two hybridomas were obtained from CIP−BSA, NOR−BSA, ENR−BSA, and the OFL−BSA conjugates, respectively. All Mabs were of the IgG1 isotype having κ light chains, and the protein concentrations of all Mabs were between 4.3−8.1 mg mL^{-1}.

Preliminary Antibody Characterization. The nine selected Mabs (Table 1) were used in a bidirectional titration assay to determine optimal immunoreagent concentrations for use in the ciELISA. Mabs C1G6B8, E2F4C5, N2H3A8, and O2A6B5 had the highest affinity to their corresponding analytes (data not shown). Since our aim was to produce an antibody with broad specificity to all FQs, 14 FQ analogs were tested against all the 9 Mabs using a screening competitive ELISA (data not shown) in order to determine the Mabs specificity. Four Mabs C1G6B8, C4A9H1, N4D5G3, and N2H3A8 demonstrated broad specificity (Table 1). The Mab N3G6C7 and the Mabs O3D4G9 and O2A6B5 showed limited recognition of the other FQs. Mab E2F4C5 recognized only ENR and CIP. Mab E5B7E8 was the only antibody that was specific to only one of the haptens (ENR) and showed negligible cross-reactivity with the other related FQs.

The results reported here are consistent with previous studies.[15,16,19,22] When ENR was used as a hapten, then primarily narrow-specificity antibodies were obtained. When CIP was used as the hapten, then more broad-specificity antibodies were obtained.

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Table 1. Parameters of Optimized ciELISA for Nine Monoclonal Antibodies with Corresponding Coating Antigens[a]

Mab	hapten	antibody concn (ng mL^{-1})	coating antigen concn (ng mL^{-1})	A_{max}	IC_{50} (ng mL^{-1})	working range (ng mL^{-1})	LOD (ng mL^{-1})	R^2
C1G6B8	CIP	33.5	10	1.837	23	5.3−221	2.4	0.997
C4A9H1	CIP	16.0	6.0	1.724	8.9	2.0−34	1.2	0.996
E2F4C5	ENR	49	3.3	1.735	2.7	0.5−14	0.2	0.998
E5B7E8	ENR	37.2	5.2	1.694	5.9	1.3−21	0.6	0.997
N4D5G3	NOR	25.3	6.0	1.812	3.0	0.8−11	0.3	0.997
N2H3A8	NOR	22.0	4.2	1.678	2.1	0.4−9	0.2	0.997
N3G6C7	NOR	25.5	8.4	1.749	19	2.3−76	3.4	0.999
O2A6B5	OFL	14.3	4.3	1.767	6.3	2.1−24	1.1	0.997
O3D4G9	OFL	20.3	4.1	1.876	3.9	1.4−13	0.8	0.997

[a] These parameters from various ciELISAs were obtained from four-parameter logistic equations used to fit the standard curves, and these results represent the mean of three separate experiments each of which contained a minimum of four replicates.

Immunoassay Optimization and Evaluation. Properties of the buffer system as well as the assay conditions are the most immediate parameters to be optimized. Also, the lowest possible coating concentration that allows reliable detection of the label and does not affect the competition is desired for highest sensitivity. Also, incubation times were other important factors that govern the sensitivity of ELISAs. A combination of the Mab C4A9H1 along with the corresponding coating antigen CIP−OVA afforded the best broad specificity for the FQs and was selected for further optimization and evaluation in the immunoassay protocol.

Coating Buffer, Blocking Buffer, Antibody Dilution Buffer, and Enzyme-Labeled Secondary Antibody Dilution Buffer. Three coating buffers, carbonate buffer, phosphate buffer, and citrate buffer, were evaluated for their influence on ELISA performance. The use of carbonate buffer (pH 9.6) as coating buffer obtained the best IC_{50} (8.6 ng mL^{-1}) and A_{max} (1.824) values, while phosphate buffer (pH 7.4) and citrate buffer (pH 6.6) both decreased A_{max} and increased the IC_{50} values when used as coating buffers. Carbonate buffer was chosen as coating buffer because it appeared to favor the absorption of OVA to the surface of the polyethylene microtiter plates.

Coating Antigen Concentration and Antibody Concentration. Concentrations of the Mab C4A9H1 (6.4 mg mL^{-1}) and coating antigen CIP−OVA (1.2 mg mL^{-1}) were optimized to provide an adequate A_{max} in the range of 1.5−2.0, in the absence of analyte. The optimal concentrations of Mab C4A9H1 and coating antigen CIP−OVA were used in a competitive assay, and the minimum absorbance (background signal) obtained with excess analyte was approximately zero. Variation of coating antigen concentrations from 4 to 12 ng mL^{-1}, in combination with antibody concentrations from 8 to 12.8 ng mL^{-1}, produced a variation of IC_{50} values from 41.5−8.9 ng mL^{-1}. A CIP−OVA coating at 6 ng mL^{-1} and an antibody concentration at 16 ng mL^{-1} were the best immunoreagent concentrations for the immunoassay producing the lowest IC_{50} for CIP (8.9 ng mL^{-1}) and an adequate A_{max} (1.724) (Table 1).

Incubation Time and Stop Time. Incubation time periods of around 30−60 min have been chosen for the competitive step in many ELISA protocols.[38,39] Then following the competitive step, the enzyme-labeled secondary antibody was added to the reaction system. At least 30 min of incubation time was required for the enzyme−substrate reaction, and sometimes an incubation time of 2 h was required to maintain an appropriate assay signal and sensitivity. In an attempt to reduce the time of this CIP ciELISA, the antibody, analyte, and enzyme-labeled secondary antibody were simultaneously added to the well and incubated for 1 h. With this strategy, it was proposed that the incubation time for the competitive step between the analyte and antibody was 0 min. The CIP and Mab were incubated in the CIP−OVA-coated plates at different periods of time (0, 15, 30, and 60 min) before adding the enzyme-labeled secondary antibody. In this case, no significant variation was observed in the IC_{50} and recovery values of 20 ng g^{-1} CIP spiked chicken muscle, but there was a decreased assay signal (A_{max}). The IC_{50} values were 8.8, 9.4, 8.7, and 8.6 ng mL^{-1}, and chicken muscle recoveries at 20 ng g^{-1} CIP were 87%, 84.5%, 84%, and 86%, respectively. However, reducing the competition time from 60 to 0 min resulted in a decrease of A_{max} from 2.4 to 1.75. Despite a 27% reduction in assay signal, the strategy was used because of the reduced time of manipulation, while still maintaining good sensitivity and recovery. Also, the reaction time between the substrate and enzyme has an influence on the ciELISA. Different time periods (5, 15, 30, and 45 min) before the addition of the stop buffer were investigated. Stopping the assay at or before 15 min resulted in a recovery of 62% for CIP from the chicken muscle and an increased background. However, stopping the enzyme reaction at 30 min resulted in an 89.5% recovery of CIP. Therefore, 30 min was selected as the optimum stop time.

The eight other ciELISAs using the respective eight Mabs for the determination of CIP, NOR, ENR, and OFL were optimized with the same conditions as was the CIP ciELISA using Mab C-4A9-H1. Table 2 shows that all Mabs had strong affinity for their corresponding BSA-conjugated FQs. The Mab N-2H3-A8 provided the highest sensitivity (IC_{50} of 2.1 ng mL^{-1} and LOD of 0.2 ng mL^{-1}) for NOR.

Immunoassay Specificity. The specificity of the 9 antibody clones was evaluated by performing competitive assays using 14 FQs, and the obtained IC_{50} values (ng mL^{-1}) were used to calculated cross-reactivity. The results are summarized in Table 2. The values represent the mean of three separate experiments each of which contained a minimum of four replicates. All of the

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Table 2. The IC_{50} and Cross-Reactivities (CR) of the FQs in the Nine ciELISAs[a]

	cell clones																	
	C1G6B8		C4A9H1		E2F4C5		E5B7E8		N4D5G3		N2H3A8		N3G6C7		O2A6B5		O3D4G9	
FQ	IC_{50} ng mL^{-1}	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %	IC_{50} ppb	CR %
CIP	23	100	8.9	100	3.3	82	293	2	6.1	49	3.6	58	79	24	12.5	16	485	0.01
ENR	34	67	10.9	82	2.7	100	5.9	100	6.0	50	3.4	62	70	27	18	11	63.5	0.06
NOR	31	75	12.5	71	>500	<0.01	>500	<0.01	3.0	100	2.1	100	19	100	10.4	38	5.9	66
OFL	68	34	16.5	54	>500	<0.01	>500	<0.01	5.2	58	3.3	63	62	31	6.25	100	3.9	100
DAN	46	50	16.5	54	>500	<0.01	>500	<0.01	6	50	3.4	62	>2500	<0.01	99	0.06	113	0.03
PEF	19	121	18	49	>500	<0.01	>500	<0.01	3.7	81	2.2	100	>2500	<0.01	70	0.1	82.5	0.05
AMI	100	23	17.5	51	>500	<0.01	>500	<0.01	4.6	65	3.3	63	>2500	<0.01	127.5	0.05	47.5	0.08
LOM	192	12	22	40	>500	<0.01	>500	<0.01	9.1	33	4.4	48	>2500	<0.01	230	0.03	197	0.02
ENO	128	18	20	45	>500	<0.01	>500	<0.01	5.1	59	3.3	63	>2500	<0.01	92.5	0.08	93	0.04
FLU	329	7	25.5	35	>500	<0.01	>500	<0.01	9.6	31	3.9	54	>2500	<0.01	345	0.02	48	0.08
OA	383	6	22	40	>500	<0.01	>500	<0.01	4.7	64	3.3	63	>2500	<0.01	119	0.05	210	0.02
MAR	110	21	21.5	41	>500	<0.01	>500	<0.01	4.9	61	3.7	57	>2500	<0.01	67.5	0.1	65	0.06
DIF	>5000	<0.01	284	3.1	>500	<0.01	>500	<0.01	920	<1	909	<0.01	>2500	<0.01	500	0.01	500	0.01
SAR	>5000	<0.01	307	2.9	>500	<0.01	>500	<0.01	953	<1	959	<0.01	>2500	<0.01	500	0.01	500	0.01

[a] The values of IC_{50} were obtained from four-parameter logistic equations used to fit the standard curves, and these results represent the mean of three separate experiments each of which contained a minimum of four replicates.

antibodies demonstrated the highest affinity to the hapten they were produced from except for Mab C1G6B8 (Table 1), which exhibited a higher cross-reactivity to PEF (121%) than to CIP (100%). The Mab E-5B7-E8 was highly specific to ENR, and the cross-reactivity was only 2% against CIP and well below that (0.01%) with other FQs. The ethyl group on the piperazinyl ring was important for the antibody Mab E-5B7-E8 recognition of ENR. ENR contains the only piperazinyl ring ethyl group among all the FQs tested. In the case of the Mab E2F4C5, there was an improved cross-reactivity with CIP (82%). The lack of an ethyl group on the piperazinyl ring was not detrimental to antibody binding of the cyclopropyl group at position 1 in ring B and was determined by comparing the cross-reactivity of DAN (<0.01%), which also contains a cyclopropyl ring at position 1 of ring B. Although both Mabs O2A6B5 and O3D4G9 were obtained against the conjugate OFL−BSA, and had similar cross-reactivity, Mab O3D4G9 exhibited much lower affinity for CIP (0.01%) than did Mab O2A6B5 (16%). Also, Mab O3D4G9 had a lower affinity for ENR (0.06%) than did Mab O-2A6-B5 (11%). However, Mab O3D4G9 demonstrated approximately a 2-fold higher affinity for NOR (66%) than did Mab O2A6B5 (38%). Also, both of these Mabs had low affinity for other FQ analogs with cross-reactivity below 0.1%. It is difficult to explain why both Mabs showed a higher affinity for NOR and lower affinity for both PEF and MAR, while MAR looks more structurally similar to OFL than to NOR. As discussed in the Haptens section, broad-spectrum antibodies were produced using the NOR hapten, two of the three Mabs produced to NOR exhibited high affinity to most FQs, but the Mab N3G6C7 had lower affinity for NOR.

Although Mab N-2H3-A8 exhibited the lowest IC_{50} values for FQ analogs, Mab C4A9H1 exhibited the greatest broad cross-reactivity with all 14 FQs tested (including SAR and DIF), and this cross-reactivity data was used to analyze the effect on antibody binding and the FQ structures with molecular modeling. Figure 1 demonstrates that these molecules can be roughly divided into four groups. The first group contains the four drugs CIP, ENR, NOR, and PEF and possess the most similar structures with the hapten containing the piperazinyl ring at position 7 of ring A and an alkyl group at position 1 of ring B. The second group consists of five drugs OFL, ENO, MAR, LOM, and AMI. Although they contain the piperazinyl ring at position 7 of ring A, some heteroatom structures exist in these molecules, such as the nitrogen at position 8 in ring A of ENO. The third group, consisting of DAN, OA, and FLU does not have a piperazinyl ring. The fourth group consists of two drugs, SAR and DIF, and both contain a fluorophenyl group at position 1 of ring B. Inspection of the IC_{50} values provide clues as to which determinant groups are important for binding the Mab C4A9H1. The effect on antibody binding on the type of substitute groups on the central FQ moiety can be determined by comparing the various FQs to CIP or to each other.

A comparison of the IC_{50} values of the FQs lacking the cyclopropyl group at position 1 of CIP demonstrates that the loss of this group and substitution with other groups has a diverse degree of effects on binding. The deleterious effect on antibody binding caused by the fluorophenyl group at position 1 can be seen by comparing the IC_{50} values of Mab C4A9H1 for CIP (8.9 ng mL^{-1}) to the IC_{50} values for SAR (307 ng mL^{-1}) and DIF (284 ng mL^{-1}). Approximately, a 33-fold increase in the IC_{50} value occurs as the result of changing the cyclopropyl group to a fluorophenyl group. The structure of NOR is similar to that of SAR in all respects except for the ethyl group, which is replaced by the fluorophenyl group at position 1 in SAR. Only a 1.2-fold increase in the IC_{50} value was observed for NOR compared to CIP. Similarly, an analogous comparison of ENR to DIF results from an alkyl substitution attached to the secondary amine in the piperazinyl ring and a cyclopropyl group substituted by a fluorophenyl group in DIF, and these substitutions produce a 26-fold increase in the IC_{50} value for DIF compared with that of ENR (Figure 1). This was not surprising since the presence of the fluorophenyl group introduces a larger substituent at position 1 of ring B in SAR and DIF than in CIP, and the aromatic ring as well as the electronegative fluorine would be expected to involve a number of binding interactions. The importance of the cyclopropyl group or the introduction of an ethyl group at position 4 of the piperazinyl ring to antibody binding can be determined by

comparing the IC_{50} value for CIP (8.9 ng mL^{-1}) to the IC_{50} value of NOR (12.5 ng mL^{-1}) or ENR (10.9 ng mL^{-1}) (Table 2 and Figure 1). It is thought that changing the substituents at these positions has little effect on binding. The combined effect of changing the group at position 1 and at the piperazinyl ring can be evaluated by comparing the IC_{50} value for CIP to the IC_{50} values of AMI (17.5 ng mL^{-1}), PEF (18 ng mL^{-1}), and DIF (284 ng mL^{-1}) (Table 2). These results show the importance of spatial volume of the group at position 1 for antibody recognition. It was also observed that the substitution of carbon or hydrogen at position 8 in ring A with either fluorine or oxygen showed a limited effect on antibody binding by comparing the IC_{50} values of ENR (10.9 ng mL^{-1}) or PEF (18 ng mL^{-1}) to the IC_{50} values of ENO (20 ng mL^{-1}), LOM (22 ng mL^{-1}), MAR (21.5 ng mL^{-1}), and OFL (16.5 ng mL^{-1}) (Table 2). The substitution of the piperazinyl ring by a dinitrogen six-membered ring found in DAN contributed to only a 2-fold decrease in antibody binding compared to CIP. It is interesting that such a major change at this position would not cause more of an effect on binding. It maybe a result of other structural changes within DAN (i.e., substitution of a nitrogen adjacent to position 7 in ring A and the same chemical structure as CIP at position 7) providing beneficial binding opportunities for Mab C4A9H1 that offset the ring substitution at position 7. It can be deduced that the piperazinyl ring is not an important group for antibody binding with Mab C4A9H1. There was similar cross-reactivity observed with the antibody against SAR and with trovafloxacin, which closely resembles DAN because it has a similar group as DAN at position 7.[18] The cross-reactivity of FLU compared to CIP was unexpected, with both the loss of the piperazinyl ring and substitution of the cyclopropyl group by the 1-oxo-1H,5H-benzo(*ij*) quinolizine group resulting in FLU being structurally much different and leaves only the core quinolonecarboxylic acid intact. These large changes result in only a 2.9-fold decrease in antibody binding compared with that of CIP. More surprisingly, in the case of OA, the loss of the piperazinyl ring and the substitution of fluorine by an oxygen at position 6 only exhibited a 2.5-fold decrease in antibody binding compared with that of CIP; however, the results we observed are consistent with that of the NOR antibody, which has 40% cross-reactivity with OA, the highest cross-reactivity of all nine FQs except for that of ENO as seen in another study.[27] The cross-reactivity of Mab C4A9H1 with OA and FLU supports the hypothesis above that the piperazinyl ring at position 7 in CIP is not as important as other sites in binding, since the Mab showed high cross-reactivity (35−82%) with most of the FQs tested in the study. The lowest cross-reactivity was observed when SAR and DIF were competitors, demonstrating that the group at position 1 representing a larger aromatic structure such as the benzene ring exerts a large effect on antibody binding.

It is believed that the hapten− or antigen−antibody interaction is dependent on molecular shape, is defined by the geometry, and on low-energy interactions such as hydrogen bonding, hydrophobic interactions, and electrostatic and dipole−dipole forces together with $\pi-\pi$ complementary ring-bonding.[34] Inspection of the 2D structures of the FQs could not explain the ELISA data and which forces mainly contribute to the Mabs binding with these FQs. In an effort to determine which structural and electronic effects were primarily important for antibody−FQ binding, further studies using advanced molecular modeling techniques were undertaken.

Molecular Modeling Studies. Molecular modeling can provide useful information regarding physicochemical properties of chemicals and can assist in designing haptens.[40,41] However, most often molecular modeling is used as a tool to explain the cross-reactivity of an antibody.[37,42,43] Computer molecular modeling is used here to conduct studies on the compounds shown in Figure 1 to correlate their conformational and electronic properties with their ability to cross-react with the Mab C4A9H1.

Lowest Energy Conformations. These 3D structures are shown in Figure 2, which displays the global minimum energy structures of the FQs in the same orientation as in Figure 1. For all FQs, ring A and ring B lie in the same plane, except for FLU, where ring A bends downward and forms an angle with ring B, which may be responsible for a 75% decrease in the IC_{50} value compared to that of CIP. The first group contains four drugs CIP, ENR, NOR, and PEF, which are the most conformationally and chemically similar at both position 1 and position 7. The introduction of an ethyl in ENR and a methyl in PEF on the piperazinyl ring (as compared to no group in the piperazinyl ring in CIP and NOR) introduces a perturbation in that ring but does not affect the superimposability of the piperazinyl ring. Also, replacement of the cyclopropyl ring by an ethyl group in NOR does not affect the planarity of the molecule (compared to the structure of CIP). Although substitutions can occur at position 1 and 2 of the piperazinyl ring as seen in OFL, ENO, MAR, LOM, and AMI, the 3D structures of these drugs are consistent with that of CIP, and the antibody also exerts similar cross-reactivity with these drugs (41−54%).

The third group consists of DAN, OA, and FLU; the conformation of DAN demonstrates that substitution of the piperazinyl ring as found in CIP with the bicyclic ring does not dramatically change the general conformational structure as seen by the antibody. Even though the chemical is quite different, mimicry of the conformational structure of CIP may explain why the Mab binds DAN with about half the CR (54) as CIP. Moreover, the other drugs, OA and FLU, both have a loss of the piperazinyl ring at position 7 resulting in an entirely different conformational structure and lower antibody affinities of 40 and 35, respectively. This result points out that the substituents on ring B are more important for binding with this antibody.

The fourth group is SAR and DIF, and in respect to CIP this group has steric hindrance due to the larger fluorophenyl ring at position 1. In Figure 2, the plane formed by the fluorophenyl ring lies almost perpendicular to the plane formed by ring A and ring B. The lower binding affinity exhibited by Mab C4A9H1 for SAR and DIF reflects the introduction of this steric hindrance.

Electronic Properties. Figure 3 shows the electrostatic potential displayed over the electron density surfaces of the global minimum energy conformations of the FQs. The charges of the isosurfaces are color coded as shown in the legend of Figure 3. The drugs in

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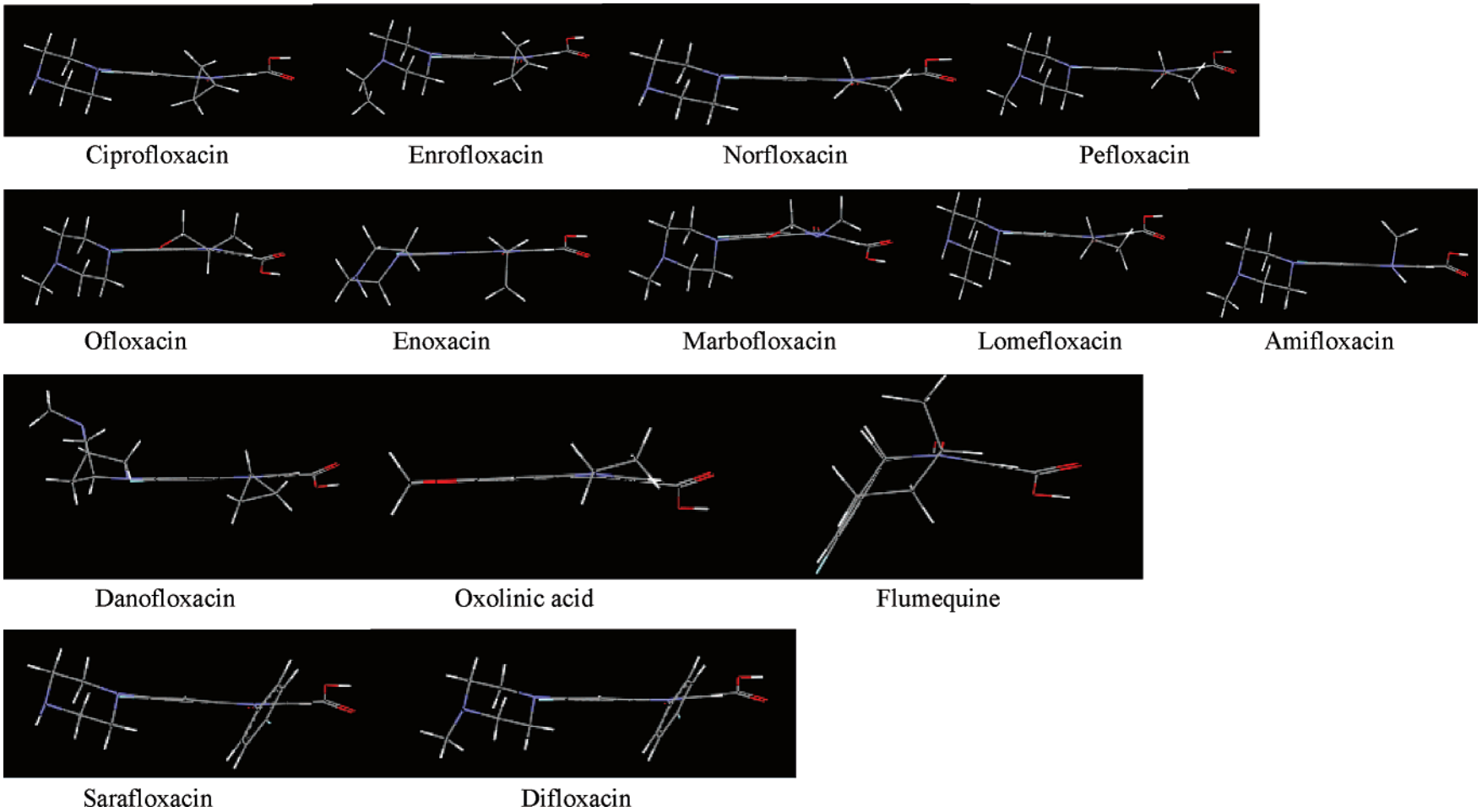

Figure 2. Models of the minimum energy conformations of the FQs. The elements are represented in the following manner: oxygen, red; nitrogen, blue; hydrogen, white; fluorine, yellow; carbon, off-white.

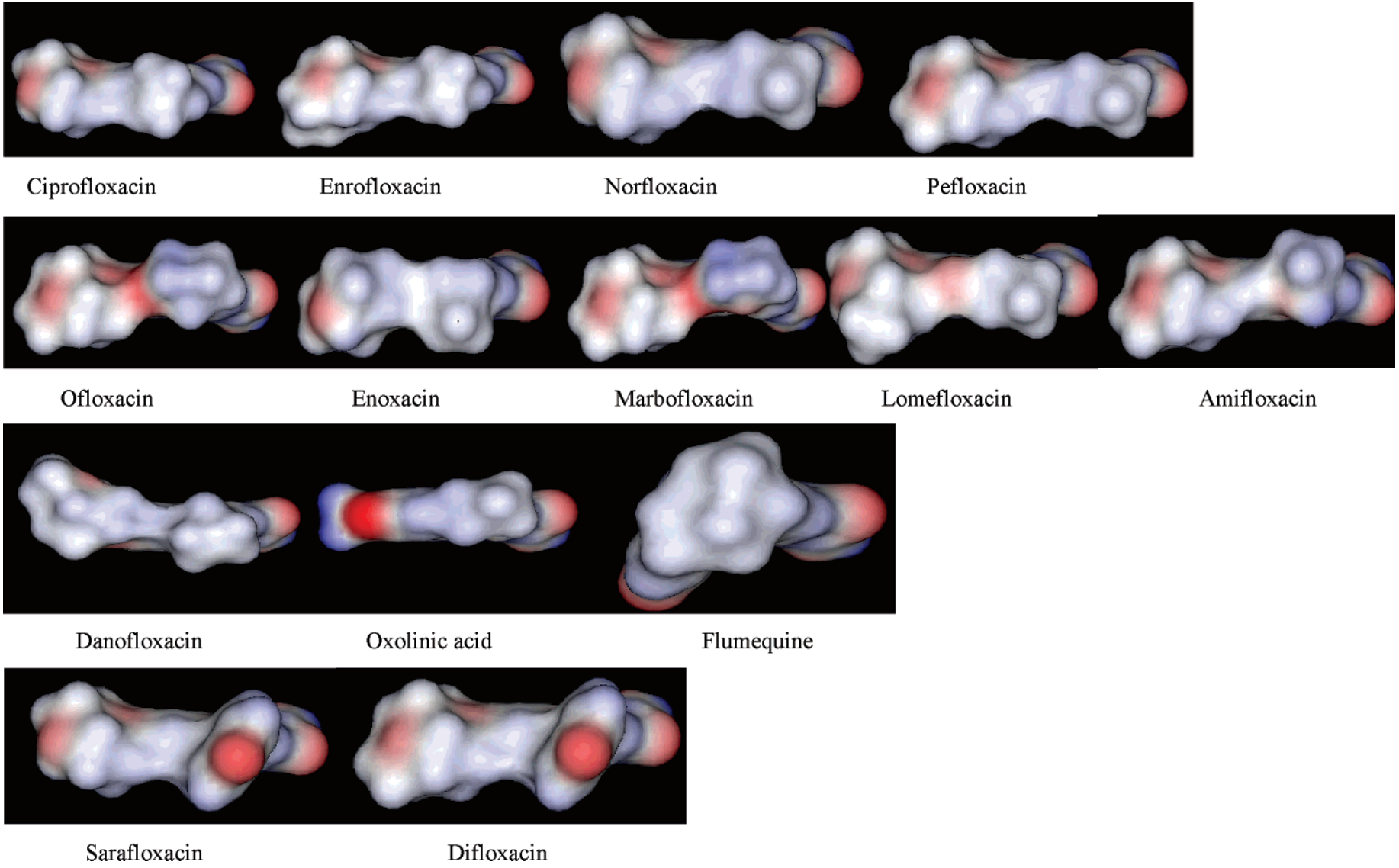

Figure 3. Electrostatic potential overlaid on the electron density isosurfaces of the minimum energy conformations of the FQs. Red, negative charge; blue, positive charge; white, neutral charge.

the first group have almost identical surface charges, which are consistent with the antibody binding characteristics (Figure 3 and Table 2). In contrast, the drugs in the second group, OFL, ENO, MAR, LOM, and AMI, show greater similarity in surface charge and structure on the portion of the molecules containing the piperazinyl ring, the fluorine atom at position 6, and carboxyl at position 3. The drugs in groups 1 and 2 differ by a negative surface region in the second group corresponding to the electronegative atom (the red regions). The shapes of the drugs in the third group are much different than that of CIP, except for DAN. The nitrogen in the bicyclic ring system of DAN is bent out of the plane of the molecule compared to the piperazinyl ring of CIP, and this change results in a 46% decrease in the IC_{50} value. Although the loss of the piperazinyl ring in OA results in a substantial chemical structural difference compared to that of CIP, the introduction of the bioxygen ring at position 7 offsets the loss of the nitrogens on the piperazine ring and the fluorine at position 6 and are reflected in only a 60% decrease in IC_{50} value. However, even though there is a distinct difference in the structure of FLU compared to that of CIP, the antibody shows good affinity to FLU. The higher cross-reactivity demonstrates that the linear shape of the drug is not necessary for antibody binding. Here we see again that the antibody appears to primarily bind features around ring B. The same case was observed with the fourth group. The replacement of the cyclopropyl ring by the fluorophenyl ring not only introduced steric but electronic effects resulting in about a 30-fold decrease in IC_{50} values.

CoMFA Analysis. CoMFA is a widely used technique for the study of the quantitative structure−activity relationship (QSAR) at the 3D level. The 3D-QSAR/CoMFA analysis generates steric and electrostatic contour maps in 3D space around the molecule in respect to changes in activity.[44] The IC_{50} values of 14 structurally related FQ analogs used as competitors in an ELISA using the Mab C4A9H1 and CIP−OVA as the coating antigen (Table 2) were submitted to CoMFA analysis. The CoMFA model obtained showed a high predictive ability with a cross-validation q^2 value of 0.866. The non-cross-validated r^2 value was 0.999 and F test value was 1851.251. The contributions of the steric and electrostatic fields to activity, as indicated by IC_{50} values, were 58.1 and 41.9% by PLS analysis, respectively. This result means that the steric factor for groups or atoms plays more of an important role on antibody affinity for the FQ analogs than does the electrostatic factor. Figure 4 is a contour map that shows where the changes in molecular and electrostatic fields are associated with the differences in antibody binding to CIP. Greater values were correlated with more bulk near the green contours, less bulk near the yellow contours, more positive charge near the blue contours, and more negative charge near the red contours. Figure 4 shows that antibody affinity would be restricted by the presence of bulky groups in the region of the yellow-colored contours, which are near position 1, position 8, and the piperazinyl ring at position 7, while bulky groups near the green-colored contours (around the fluorine atom at position 6) increased antibody affinity. Also, a positive charge near the blue contour regions and a negative charge near the red contour regions favor increased antibody affinity.

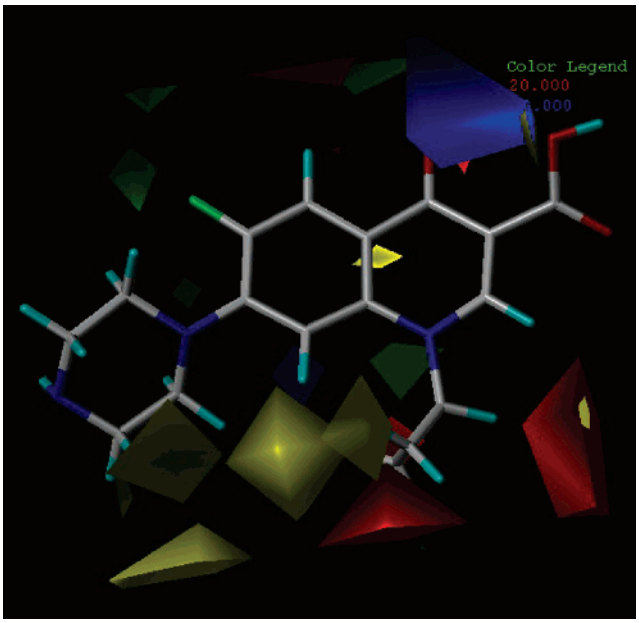

Figure 4. Contour plots of CoMFA steric and electrostatic fields. Green contours indicate regions where bulky groups increase antibody affinity, and yellow contours indicate regions where bulky groups decrease antibody affinity. Blue contours indicate regions where positively charged groups increase antibody affinity, and red contours indicate regions where negatively charged groups increase antibody affinity.

Recovery Study. Five different matrices were chosen for an ELISA recovery evaluation. The performance of the extraction method was evaluated for chicken muscle, chicken liver, shrimp, honey, and whole egg. Because FQs are soluble in polar organic solvents but not in nonpolar solvents, and are soluble in hydro−organic or aqueous acidic and basic buffer, methods usually blend polar organic or aqueous−organic solvents and utilize solid-phase extraction (SPE) as a purification technique. However, cleanup involving ion-exchange, adsorption, or reversed-phase extraction are time-consuming, expensive, and can be reproduced with varying degrees of success, which is strongly influenced by the manufacturer/batch of SPE column used.[45] Several authors have reported a simple extraction process for FQs used in an ELISA.[15,18,22] In these extraction methods, FQs were extracted with only assay buffer and the extracts had no further purification. In our work, dichloromethane and (or) acetonitrile were utilized to extract six FQs (ENR, CIP, NOR, OFL, FLU, and DAN) from five matrices (chicken muscle, chicken liver, honey, shrimp, and whole egg). For each matrix, known negative samples spiked with FQs at 50, 100, and 200 ng g^{-1} were extracted and analyzed using the optimized ELISA procedure. The results are shown in Table 3. Recovery for each of the six FQs was observed in the range of 60−93%. The precision (% CV) between analyses remained below 16%.

In the specificity section, the generic ELISA was demonstrated to detect 12 among all 14 FQs tested below the maximum residue levels (MRLs) except for DIF and SAR, which have a low cross-reactivity of 3.1% and 2.9%, respectively. The six FQs (CIP, ENR, NOR, OFL, FLU, and DAN) largely used in the animal industry

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Table 3. Recovery of Six FQs from Five Fortified Food Samples

		ENR			CIP			NOR		
sample	compds added ng g^{-1}	found ng g^{-1}	% recovery	CV	found ng g^{-1}	% recovery	CV	found ng g^{-1}	% recovery	CV
chicken muscle	50	34.1	68.2	12.3	30.7	61.4	8.6	88.5	87.0	12.4
	100	62.5	62.5	16.4	69.7	69.7	7.5	86.4	86.4	8.5
	200	140.8	70.4	13.4	139.0	69.5	13.2	153.0	76.5	7.9
chicken liver	50	31.9	63.7	11.4	30.3	60.8	5.6	39.1	78.2	8.5
	100	66.5	66.5	9.4	74.2	74.2	9.4	76.4	76.4	5.9
	200	139.6	69.8	9.5	142.8	71.4	13.2	151.0	75.5	12.7
shrimp	50	32.9	65.8	13.5	32.7	65.4	10.5	43.3	86.6	10.5
	100	67.4	67.4	10.8	65.8	65.8	11.6	89.4	89.4	9.4
	200	164.8	82.4	16.4	163.0	81.5	8.5	170.8	85.4	10.5
honey	50	36.6	73.2	11.4	30.2	60.4	10.5	30.2	60.4	13.4
	100	60.4	60.4	9.4	72.1	72.1	14.6	62.3	62.3	10.8
	200	136.8	68.4	16.2	125.0	62.5	13.4	120.4	60.2	16.5
whole egg	50	34.2	68.5	10.4	36.0	72.1	6.7	35.7	70.4	10.7
	100	78.6	78.6	8.3	63.4	63.4	18.2	62.5	62.5	8.4
	200	149.4	74.7	6.4	134.8	67.4	12.4	139.2	70.6	6.9

		OFL			FLU			DAN		
	compds									
chicken muscle	50	33.7	67.4	10.5	32.4	64.8	7.5	42.1	84.1	13.7
	100	68.1	68.1	10.9	72.5	72.5	9.4	82.6	82.6	14.6
	200	165.0	82.5	14.2	138.8	69.4	7.5	185.0	92.5	7.6
chicken liver	50	36.3	72.5	8.6	342.0	68.3	6.4	141.8	70.9	12.4
	100	65.4	65.4	7.5	65.1	65.1	8.2	63.4	63.4	9.5
	200	159.6	79.8	15.4	123.0	61.5	13.7	156.8	78.4	6.7
shrimp	50	31.4	62.8	7.6	37.0	74.0	12.3	41.4	82.7	12.7
	100	64.5	64.5	13.5	64.9	64.9	7.5	74.9	74.9	14.6
	200	142.8	71.4	9.6	159.6	79.8	15.2	145.0	72.5	13.4
honey	50	31.0	62.1	8.4	34.2	68.4	8.4	31.2	62.4	10.7
	100	78.4	78.4	12.4	72.0	72.0	15.0	60.7	60.7	13.4
	200	167.0	83.5	11.6	146.4	73.2	14.5	152.8	76.4	14.2
whole egg	50	34.2	68.4	7.2	37.7	75.4	8.4	40.2	80.4	8.6
	100	70.4	70.4	9.7	62.4	62.4	8.6	71.4	71.4	15.4
	200	139.0	69.5	14.5	121.6	60.8	15.4	125.0	62.5	13.0

and aquaculture in China were selected for a more detailed study. Although other FQs (PEF, AMI, LOM, ENO, OA, and MAR) also had good cross-reactivity with CIP, they were not further evaluated here. However, on the basis of preliminary data it can be speculated that the ELISA developed here could be capable of detecting 12 FQ residues in the 5 matrices tested.

CONCLUSION

We report here several Mabs with high cross-reactivity to 12 FQs. The selection of the right hapten is the key step for the whole process of producing broad-specificity antibodies. In all papers describing the production of FQ antibodies, the authors have the same general view that the FQs not containing a substituent on the piperazinyl ring make the best haptens for producing a broad-specificity antibody, and most of the antibodies obtained from these haptens were of the generic nature. Considering the structural features of the FQs and the cross-reactivity data provided in previous studies, CIP and NOR were selected as the optimum haptens to produce a broad-specificity antibody. The Mabs raised against these two haptens demonstrated broad specificity against 12 of the 14 FQs tested; however, none of the Mabs obtained recognized two important FQs, SAR and DIF. Previously we mentioned that the antibodies raised against the haptens CIP and ENR, which have similar structures, showed an unusual cross-reactivity as they did in other studies.[16,19] The cross-reactivity data obtained here demonstrates that the ethyl group of the piperazinyl ring exerts limited effect on antibody binding, but this group appears to be important during antibody production. In the case of another steric domain, position 1 (Figure 1) emerges as important for antibody generation. SAR has a structure that closely resembles that of CIP but contains a larger fluorophenyl ring at position 1. From our cross-reactivity data (Table 2), it was observed that the introduction of steric hindrance at position 1 is the most important factor in reducing antibody binding, but steric hindrance at the site seems not to be important during antibody

generation. Our hypothesis has also been verified by evaluating other previous studies. The anibodies[18,22] raised against SAR exhibited high cross-reactivity with SAR, DIF, and other FQs, while antibodies raised against NOR or CIP exhibited much lower cross-reactivity with SAR.[29] Therefore, perhaps in the future SAR might be the best hapten to use to produce antibodies having broad specificity.

The IC_{50} data of Mab C4A9H1 binding to 12 FQ analogs were directly put into the CoMFA model analysis to investigate the relationship of antibody affinity with the structural features of the FQ analogs. The calculated results demonstrate that the 3D-QSAR method is useful in analyzing cross-reactivity data; moreover, it can predict antibody affinity for analogs that are not in the study. Finally, a ciELISA was developed for the determination of six FQs commonly used in the animal industry, and recovery results in five different foods demonstrated that these antibodies can be used in multiresidue monitoring of FQs in food samples.

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